LABORATORY NAME:		CERT #:	
PRIMARY ANALYST:		DATE:	
NAME OF PERSON COMPLETING CHECKLIST (PRINT):			
SIGNATURE OF PERSON COMPLETING CHECKLIST:			

Parameter: **Thermotolerant (Fecal) Coliform - Membrane Filtration Procedure**
Method: **SM 9222 D-2006 (MF) (Aqueous)**

EQUIPMENT: Not all items are required.

	Water bath incubator, 44.5 ± 0.2ºC (calibrated or accurate to ± 0.1°C accuracy) with gabled cover		Autoclave
	pH meter with solid surface probe for agar		Temperature gauge
	Thermometer 0.1°C increments		• Pressure gauge
	Graduated cylinders		• Holding thermometer
	Sterile pipettes		• Vacuum source
	Refrigerator		Sterile non-leaking filtration apparatus
	Microscope w/ 10-15x magnification or other optical device		Colony counter
	Forceps		Verification equipment
	Sterilizer oven 170ºC		Incubator, 35.0 ±0.5°C (air or water)
	Hotplate w/ magnetic stirrer		• Fermentation tubes
	Maximum Registering Thermometer		• Durham tubes
	Bunsen Burner (or flame source) with alcohol to flame		• Inoculating equipment
	Dilution bottles		

CONSUMABLES: Not all items are required

	Waterproof plastic bag enclosures		Petri dishes
	Sterile absorbent pads		Sterile membrane filters, 0.45 µm

REAGENTS: Not all items are required

	M-FC broth		Sterile dilution/ rinse water
	M-FC agar		• Phosphate buffer (KH_2PO_4)
	1% Rosolic Acid		• $MgCl_2$
	0.2 *N* NaOH		Lauryl Tryptose Broth (LTB)
	10% $Na_2S_2O_3$		EC Medium

PLEASE COMPLETE CHECKLIST IN INDELIBLE INK
Please mark Y, N or NA in the column labeled LAB to indicate the common lab practice and in the column labeled SOP to indicate whether it is addressed in the SOP.

	GENERAL	LAB	SOP	EXPLANATION
1	Is the SOP reviewed at least every 2 years? What is the most recent review/revision date of the SOP? [15A NCAC 2H .0805 (a) (7)] **ANSWER:**			Quality assurance, quality control, and Standard Operating Procedure documentation shall indicate the effective date of the document and be reviewed every two years and updated if changes in procedures are made. **QC is 9020 A, B, and C in SM 22nd Edition – 2005. 9030-9060 is found in 22nd Edition – 2006.** **SM 9020 A-2005 states: QC requirements in section 9020 are not mandatory.** Each laboratory must develop its own QC suitable for its needs and, in some cases, as required by regulatory agencies, standard setting organizations, and laboratory certification or accreditation programs. The program must be practical and require only a reasonable amount of time or it will be bypassed. Once a QA program is established, about **15%** of overall laboratory time should be spent on different aspects of the program. When properly administered, a balanced,

				conscientiously applied quality system will optimize data quality, identify problems early, and increase satisfaction with the analytical results without adversely affecting laboratory productivity. SM 9020 A-2005 (4) states: The QC guidelines discussed in 9020 B and 9020 C are recommended as useful source material of elements that need to be addressed in developing policies for a QA program and QC activities. Based upon this language, in conjunction with method specified requirements, the NC WW/GW LC program has established minimum requirements for maintaining certification from our program. These are addressed in this checklist along with recommendations to be considered as the laboratory's QC program evolves over time. Verify proper method reference. During review notate deviations from the approved method and SOP.
2	Are all revision dates and actions tracked and documented? [15A NCAC 2H .0805 (a) (7)]			Each laboratory shall have a formal process to track and document review dates and any revisions made in all quality assurance, quality control and SOP documents.
3	Is there North Carolina data available for review?			If not, review PT data.
	PRESERVATION and STORAGE	**LAB**	**SOP**	**EXPLANATION**
4	Are samples collected in sterile containers? [SM 9060 A-2006 (1) and 40 CFR Part 136.3 Table II]			Collect samples for microbiological examination in clean, sterile, nonreactive borosilicate glass or plastic bottles (i.e., any plastic that is made of a sterilizable material such as polypropylene or other autoclavable plastic) or presterilized plastic bags appropriate for microbiological use. Sterilize as directed in section 9030 B-2006 (19) and 9040-2006.
5	Is residual chlorine neutralized at time of sample collection with sterile $Na_2S_2O_3$? [SM 9060 A-2006 (2) and 40 CFR Part 136.3 Table II]			For sampling chlorinated wastewater effluents, add sufficient $Na_2S_2O_3$ to a clean sample bottle to give a concentration of 100 mg/L in the sample. In a 120-ml bottle 0.1 ml of a 10% solution of $Na_2S_2O_3$ will neutralize a sample containing 15 mg/L residual chlorine. This will yield a **0.008%** $Na_2S_2O_3$ solution as required in the 2017 MUR.
6	Are samples iced to < 10 ºC during shipment? [40 CFR 136.3 Table II]			40 CFR Part 136.3 Table II, footnote 2 allows 15 minutes for sample preservation, including thermal. This means that if a sample is received in the lab within 15 minutes it is not required to be on ice.
7	Are samples checked for residual chlorine upon receipt in the lab? [40 CFR 136.3 Table II]			Use of TRC strips is allowed, see "Required Documentation for Sample Preservation and Hold Time Policy (10/30/2014)". Mix thoroughly prior to checking for chlorine.
8	What action is taken if chlorine is present? [15A NCAC 2H .0805 (a) (7) (M)] **ANSWER:**			If another sample cannot be collected, dechlorinate the sample and notify NC WW/GW Laboratory Certification group that a non-compliant sample was received. Reported results must be qualified.
9	Are samples stored at < 10 ºC prior to analysis? [40 CFR 136.3 Table II]			
	MEDIA	**LAB**	**SOP**	**EXPLANATION**
10	How is the sterile rinse/dilution water prepared? [SM 9050 C-2006 (1) (a)] **ANSWER:**			Add 1.25 mL stock Phosphate buffer solution and 5.0 ml magnesium chloride stock solution to 1-L reagent grade water. 100 ml volumes or less autoclave for 15 minutes. Rinse water volumes >100 ml adjust autoclave time for volume – see table 9020:IV SM 9020 B-2005. Final pH should be 7.2 ± 0.1. Recommended but not required to check pH. Recommend checking if performing troubleshooting

				due to suspected issues. Note that pH values will change with time. Store under refrigerated conditions after opening and discard if turbidity develops. Use within 6 months. If dilutions are prepared – do not suspend a sample in any dilution water for more than 30 minutes at room temperature because injury, death, or multiplication may occur.
11	Are the Phosphate buffer and Magnesium Chloride stock solutions sterilized after preparation and stored in the refrigerator? [SM 9050 C-2006 (1) (a)]			Stock Phosphate buffer solution; Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 ml reagent grade water, adjust to pH 7.2 ± 0.5 with 1N NaOH and dilute to 1 L with reagent grade water. Sterilize by filtration or autoclave. Store stock solution under refrigerated conditions and discard if turbidity develops. Magnesium chloride stock solution: Add magnesium chloride (38 g/L $MgCl_2$ or 81.1 g $MgCl_2$ - $6H_2O$) to 1 l reagent grade water. Sterilize and store stock solution under refrigerated conditions, discarding if solution becomes turbid.
12	Is the stock phosphate buffer documented to be pH 7.2 ± 0.5? This is considered pertinent information. [SM 9050 C-2006 (1) (a)] and [15A NCAC 2H .0805 (a) (7) (E)]			If prepared, document in the preparation instructions or if purchased, retain manufacturer's documentation stating it is the proper pH. All analytical records, including original observations and information necessary to facilitate historical reconstruction of the calculated results, shall be maintained for five years. All analytical data and records pertinent to each certified analysis shall be available for inspection upon request.
13	Is the M-FC media purchased pre-made and ready to use or prepared in the lab? If purchased pre-made skip to question #20. **ANSWER:**			
14	If <u>prepared in the lab</u>, is the preparation documented? [SM 9020 B-2005 (5) (j) (1)]			SM 9020-2005 B (5) (j) (1) Page 9-15 states: Document preparation activities such as name of media, volume produced, format, final pH, date prepared, and name of preparer.
15	Is media prepared in clean containers that are at least twice the volume of the media being prepared? [SM 9020 B-2005 (5) (j) (1)]			
16	Is reagent grade water used in preparing media? [SM 9020 B-2005 (5) (j) (1)]			
17	Is media stirred while heating? [SM 9020 B-2005 (5) (j) (1)]			Prepare according to manufacturer's instructions: Suspend 3.7 grams of the powder in 100 mL of purified water. Add 1 mL of a 1% solution of Rosolic Acid in 0.2 N NaOH> If necessary adjust the pH to 7.4 with 1 N HCl. Heat to boiling. Do not autoclave. Cool before use. Note: For most samples, mFC medium may be used without the 1% Rosolic acid addition, provided there is no interference with background growth. Ref: 9222 D-2006 (1) (a). SM 9020 B-2005 (5) (j) (1) states: Stir media, particularly agars, while heating. Avoid scorching or boil-over by using a boiling water bath for small batches of media and by continually attending to larger volumes heated on a hot plate or gas burner. Preferably use hot plate stirrer combinations.
18	Is pH of the M-FC medium adjusted if necessary and documented to be 7.4 ± 0.2 S.U.? [SM 9222 D-2006 (1) (a)] and [SM 9020 B-2005 (5) (j) (1)]			SM 9020 B-2005 (5) (j) states: Check and record pH of a portion of each media after sterilization. Adjustment of pH will seldom be necessary when commercially

				available media are used. If needed, make minor adjustments to the pH specified in the formulation with filter-sterilized 1N NaOH or 1N HCl solutions. If the pH difference is larger than 0.5 units, discard the batch and check preparation instructions and pH of reagent water to resolve the problem. If medium is known as requiring pH adjustment, adjust pH appropriately prior to sterilization and record final pH. SM 9222 D-2006 (1) (a) states: Final pH should be 7.4 ± 0.2 S.U. Bottom line: It is required to check and document the pH of each batch of prepared media after sterilization. If the pH is not 7.4 ± 0.2 S.U. it must be adjusted to that range. If using agar, the method states to pour into petri plates and let solidify before it talks about the final pH. Therefore, they need to use a pH electrode for surface measurements, which is made to be used for solid and semi-solid samples, and analyze the agar after it solidifies.
19	What is the holding time for the prepared media? [SM 9222 D-2006 (1) (a)] **ANSWER:**			Discard unused broth after 96 h or unused agar after 2 weeks.
20	If <u>purchased ready-to-use media</u> is used with a manufacturer's expiration date that exceeds the holding time stated in SM 9020 B-2005, Table 9020: V, is the manufacturer's statement of quality to that extended time on file? [SM 9020 B-2005 (5) (j) (4)]			SM states: For prepared ready-to-use media with a manufacturer's expiration date greater than noted in the Table, have manufacturer supply evidence of media quality for that extended period of time. TABLE 9020:V. HOLDING TIMES FOR PREPARED MEDIA Medium — Holding Time Broth in screw-cap flasks* — 96 h Poured agar in plates with tight-fitting covers* — 2 weeks Agar or broth in loose-cap tubes* — 2 weeks Agar or broth in tightly closed screw-cap tubes† — 3 months Poured agar plates with loose-fitting covers in sealed plastic bags* — 2 weeks Large volume of agar in tightly closed screw-cap flask or bottle* — 3 months * Hold under refrigerated conditions 2–8°C. † Hold at <30°C.
21	Is media stored in refrigerator? [SM 9222 D-2006 (1) (a)]			SM states: Refrigerate stored media. Preferably in sealed plastic bags or other containers to reduce moisture loss, and discard unused broth after 96 h or unused agar after 2 weeks. Purchased ready-to-use media may be used until the manufacturer's expiration date. See question #19.
22	Are absorbent pads used in conjunction with M-FC medium? [SM 9222 D-2006 (2) (c)] If not, skip to Question #24.			
23	While in the culture dish, is the pad saturated with at least 2.0 ml of M-FC medium and the excess decanted from the dish? [SM 9222 D-2006 (2) (c)]			Not applicable if using agar.
24	Is agar used? [SM 9222 D-2006 (1) (a)]			
25	Is the agar preparation documented? [SM 9020 B-2005 (5) (j) (1)]			SM 9020 B-2005 (5) (j) (1) Page 9-15 states: Document preparation activities such as name of media, volume produced, format, final pH, date prepared, and name of preparer.
	VERIFICATION MEDIA	**LAB**	**SOP**	**EXPLANATION**
26	Is the LTB medium purchased ready-to-use or prepared in the lab? If purchased ready-to-use skip to question #38.			Although SM 9221 B-2006 (2) (a) provides instructions for preparing medium from individual components, a commercially prepared mix of the dehydrated medium

				must be used if prepared in the lab since it is readily available. Alternatively, the medium may be purchased ready-to-use and already dispensed into tubes with inverted vials.
27	If prepared in the lab, is the preparation documented? [SM 9020 B-2005 (5) (j) (1)]			SM 9020-2005 B (5) (j) (1) Page 9-15 states: Document preparation activities such as name of media, volume produced, format, final pH, date prepared, and name of preparer.
28	Is media prepared in clean containers that are at least twice the volume of the media being prepared? [SM 9020 B-2005 (5) (j) (1)]			
29	Is reagent grade water used in preparing media? [SM 9020 B-2005 (5) (j) (1)]			
30	Is media stirred while heating? [SM 9020 B-2005 (5) (j) (1)] and [SM 9221 B-2006 (2) (a)]			Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. SM 9020 B-2005 (5) (j) (1) states: Stir media, particularly agars, while heating. Avoid scorching or boil-over by using a boiling water bath for small batches of media and by continually attending to larger volumes heated on a hot plate or gas burner. Preferably use hot plate stirrer combinations.
31	Is sufficient medium dispensed in fermentation tubes with an inverted vial (Durham tube) to cover the inverted vial at least one-half to two-thirds after sterilization? [SM 9221 B-2006 (2) (a)]			Before sterilization, dispense-in fermentation tubes with an inverted vial (Durham tube)-sufficient medium to cover the inverted vial at least one-half to two-thirds after sterilization. Note: Medium will fill the inverted vial when sterilized. Account for this volume when dispensing media into tubes.
32	Is 0.01 g/L bromcresol purple used in place of a Durham tube in the LTB? [SM 9221 B-2006 (2) (a)]			Alternatively, omit the inverted vial and add 0.01 g/L bromcresol purple to lauryl Tryptose broth to determine acid production, an indicator of a positive result in this part of the coliform test.
33	Is LTB made using 35.6 g/L dehydrated LTB? [SM 9221 B-2006 (2) (a)]			Prepare in accordance with Table 9221:I. NOTE: Since sample is not added to the LTB (the loop is simply dipped in it) only the 1 ml inoculum instructions apply for verification. TABLE 9221:I. PREPARATION OF LAURYL TRYPTOSE BROTH Inoculum mL / Amount of Medium in Tube mL / Volume of Medium + Inoculum mL / Dehydrated Lauryl Tryptose Broth Required g/L 1 / 10 or more / 11 or more / 35.6 10 / 10 / 20 / 71.2 10 / 20 / 30 / 53.4 20 / 10 / 30 / 106.8 100 / 50 / 150 / 106.8 100 / 35 / 135 / 137.1 100 / 20 / 120 / 213.6
34	Is medium autoclaved at 121°C for 12 to 15 minutes in capped tubes? [SM 9221 B-2006 (2) (a)]			Close tubes with metal or heat-resistant plastic caps. Autoclave medium at 121°C for 12 to 15 min. Note: Cap tubes loosely and set autoclave exhaust to slow.
35	After sterilization, are inverted vials free of air bubbles? [SM 9221 B-2006 (2) (a)]			Ensure that inverted vials, if used, are free of air bubbles.
36	Is pH of the LTB medium adjusted if necessary and documented to be 6.8 ± 0.2 S.U.? [SM 9221 B-2006 (2) (a)] and [SM 9020 B-2005 (5) (j) (1)]			SM 9020 B-2005 (5) (j) states: Check and record pH of a portion of each media after sterilization. Adjustment of pH will seldom be necessary when commercially available media are used. If needed, make minor adjustments to the pH specified in the formulation with filter-sterilized 1*N* NaOH or 1*N* HCl solutions. If the pH difference is larger than 0.5 units, discard the batch and check preparation instructions and pH of reagent water to resolve the problem. If medium is known as

				requiring pH adjustment, adjust pH appropriately prior to sterilization and record final pH. SM 9221 B-2006 (2) (a) states: Medium pH should be 6.8 ± 0.2 after sterilization. Bottom line: It is required to check and document the pH of each batch of prepared media after sterilization. If the pH is not 6.8 ± 0.2 S.U. it must be adjusted to that range. Use 1*N* NaOH or 1*N* HCl that has been filtered and sterilized. If the pH is more than 0.5 S.U. outside of the specified pH, discard and determine whey (e.g., incorrect preparation or abnormal pH of reagent water).
37	Is the EC medium purchased ready-to-use or prepared in the lab? If purchased ready-to-use skip to question #46.			Although SM 9221 E-2006 (1) (a) provides instructions for preparing medium from individual components, a commercially prepared mix of the dehydrated medium must be used if prepared in the lab since it is readily available. Alternatively, the medium may be purchased ready-to-use and already dispensed into tubes with inverted vials.
38	If prepared in the lab, is the preparation documented? [SM 9020 B-2005 (5) (j) (1)]			SM 9020-2005 B (5) (j) (1) Page 9-15 states: Document preparation activities such as name of media, volume produced, format, final pH, date prepared, and name of preparer.
39	Is media prepared in clean containers that are at least twice the volume of the media being prepared? [SM 9020 B-2005 (5) (j) (1)]			
40	Is reagent grade water used in preparing media? [SM 9020 B-2005 (5) (j) (1)]			
41	Is media stirred while heating? [SM 9020 B-2005 (5) (j) (1)] and [SM 9221 E-2006 (1) (a)]			Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. SM 9020 B-2005 (5) (j) (1) states: Stir media, particularly agars, while heating. Avoid scorching or boil-over by using a boiling water bath for small batches of media and by continually attending to larger volumes heated on a hot plate or gas burner. Preferably use hot plate stirrer combinations.
42	Is sufficient medium dispensed in fermentation tubes with an inverted vial (Durham tube) to cover the inverted vial at least one-half to two-thirds after sterilization? [SM 9221 E-2006 (1) (a)]			Before sterilization, dispense sufficient medium, in fermentation tubes with an inverted vial, to cover the inverted vial at least one-half to two-thirds after sterilization.
43	Is medium autoclaved at 121°C for 12 to 15 minutes in capped tubes? [SM 9221 E-2006 (1) (a)]			Close tubes with metal or heat-resistant plastic caps. Autoclave medium at 121°C for 12 to 15 min. Note: Cap tubes loosely and set autoclave exhaust to slow.
44	After sterilization, are inverted vials free of air bubbles? [SM 9221 E-2006 (1) (a)]			Ensure that inverted vials are free of air bubbles.
45	Is pH of the EC medium adjusted if necessary and documented to be 6.9 ± 0.2 S.U.? [SM 9221 E-2006 (1) (a)] and [SM 9020 B-2005 (5) (j) (1)]			SM 9020 B-2005 (5) (j) states: Check and record pH of a portion of each media after sterilization. Adjustment of pH will seldom be necessary when commercially available media are used. If needed, make minor adjustments to the pH specified in the formulation with filter-sterilized 1*N* NaOH or 1*N* HCl solutions. If the pH difference is larger than 0.5 units, discard the batch and check preparation instructions and pH of reagent water to resolve the problem. If medium is known as requiring pH adjustment, adjust pH appropriately prior to sterilization and record final pH. SM 9221 E-2006 (1) (a) states: Medium pH should be 6.9 ± 0.2 after sterilization. Bottom line: It is required to check and document the

				pH of each batch of prepared media after sterilization. If the pH is not 6.9 ± 0.2 S.U. it must be adjusted to that range. Use 1*N* NaOH or 1*N* HCl that has been filtered and sterilized. If the pH is more than 0.5 S.U. outside of the specified pH, discard and determine whey (e.g., incorrect preparation or abnormal pH of reagent water).
46	When prepared in-house, are the LTB and EC media used within the holding times specified in Table 9020:V? [SM 9020 B-2005 (5) (j) (1) Table 9020: V]			TABLE 9020:V. HOLDING TIMES FOR PREPARED MEDIA Medium — Holding Time Broth in screw-cap flasks* — 96 h Poured agar in plates with tight-fitting covers* — 2 weeks Agar or broth in loose-cap tubes* — 2 weeks Agar or broth in tightly closed screw-cap tubes† — 3 months Poured agar plates with loose-fitting covers in sealed plastic bags* — 2 weeks Large volume of agar in tightly closed screw-cap flask or bottle* — 3 months * Hold under refrigerated conditions 2–8°C. † Hold at <30°C. NOTE: For dehydrated media, you may follow the manufacturer's instructions for preparation; however, you must follow Table 9020: V for hold times.
47	If purchased ready-to-use media is used with a manufacturer's expiration date that exceeds the holding time stated in SM 9020 B-2005, Table 9020: V), is the manufacturer's statement of quality to that extended time on file? [SM 9020 B-2005 (5) (j) (4)]			SM states: For prepared ready to use media with a manufacturer's expiration date greater than noted in the Table, have manufacturer supply evidence of media quality for that extended period of time.
	PROCEDURE	**LAB**	**SOP**	**EXPLANATION**
48	Are samples analyzed as soon as possible after collection with the start of incubation no more than 8 hours after collection? [40 CFR 136.3 Table II; footnote 22]			Sample analysis should begin as soon as possible after receipt; sample **incubation (not filtration) must be started no later than 8 hours from time of collection.**
49	Are sample volumes selected to yield counts between 20 and 60 colonies per filter? [SM 9222 D-2006 (2) (a)]			
50	Are samples adequately homogenized prior to analysis or dilution? [SM 9060 A-2006 (3)]			When the sample is collected, leave ample air space in the bottle (at least 2.5 cm) to facilitate mixing by shaking, before examination. Homogenization is important since microorganisms tend to clump together.
51	Are at least two dilutions analyzed? [SM 9222 D-2006 (2) (a)]			A minimum of two dilutions is required for characterized samples. SM states that for samples with no historical data 3 dilutions are required.
52	How are sample volumes measured? [SM 9020 B-2005 (4) (k)] and [SM 9030 B-2006 (9)] **ANSWER:**			Pipets, pipettors, graduated cylinders, or graduation marks on the filter funnel are acceptable. If pipet tips are chipped, replace. If using volumetric graduation marks on the filter funnel to measure sample volume, check accuracy of graduation marks initially using a Class A graduated cylinder or volumetric pipet. Record results. Ref: SM 9020 B-2005 (4)(k)
53	If reusable glassware is used, is the glassware checked for pH and inhibitory residues? [SM 9020 B-2005 (5) (1) and (2)] **Not required at this time.**			
54	Are sterile forceps used to place the sterile membrane filter (grid side up) on the filter plate? [SM 9222 D-2006 (2) (b) and SM 9222 B-2006 (4) (c)]			Forceps are sterilized by alcohol flaming. SM 9222 D-2006 (2) (b) refers back to the Total Coliform method - 9222 B-2006 (4) (c) for the filtration of samples. SM 9020 B-2005 (5) states: Either cover glassware or store glassware with its bottom up to prevent dust from settling inside it. Funnels and graduated cylinders may be covered in aluminum foil prior to sterilization for storage until used.
55	Is the sample filtered under partial vacuum? [SM 9222 D-2006 (2) (b) and SM 9222 B-2006 (4) (c)]			SM 9222 D-2006 (2) (b) refers back to the Total Coliform method - 9222 B-2006 (4) (c) for the filtration of samples.

56	Is the time sample filtration begins documented? This is considered pertinent information.[15A NCAC 2H .0805 (a) (7) (E)]			Needed to determine if samples are put in the incubator within 30 minutes. Need to document three times – beginning (filtration) and in incubator [these will show no more than 30 minutes elapsed between filtration and the start of incubation], and out of incubator [this will document the 24 ± 2-hour incubation time] – see questions # 36 and 38.
57	With the filter still in place, is the interior surface of the filter funnel rinsed with three 20-30 ml portions of sterile buffered dilution water? [SM 9222 D-2006 (2) (b) and SM 9222 B-2006 (4) (c)]			SM 9222 D-2006 (2) (b) refers back to the Total Coliform method - 9222 B-2006 (4) (c) for the filtration of samples. A steady flow of sterile buffered dilution water from of squeeze bottle is acceptable as long as it has been sterilized along with water and does not become contaminated during use – cover tip of bottle with aluminum foil prior to sterilization.
58	Is the prepared filter aseptically placed directly on the agar or pad with a rolling motion to avoid entrapment of air? [SM 9222 D-2006 (2) (b) and SM 9222 B-2006 (4) (c)]			SM 9222 D-2006 (2) (b) refers back to the Total Coliform method - 9222 B-2006 (4) (c) for the filtration of samples.
59	Are prepared dishes sealed in water proof containers and completely submerged upside down in a water bath? [SM 9222 D-2006 (2) (d)]			Place all prepared dishes in waterproof plastic bags or seal, invert and submerge petri dishes in water bath. Anchor dishes below water surface to maintain critical temperature requirements.
60	Are all prepared samples placed in the incubator within 30 minutes of filtration? [SM 9222 D-2006 (2) (d)]			Place all prepared cultures in the water bath within 30 minutes after filtration. To meet the need for greater temperature control use a water bath, a heat sink incubator, or a properly designed and constructed incubator to give equivalent results.
61	Is the time samples are placed in the incubator documented? [15A NCAC 2H .0805 (a) (7) (F)]			The date and time that samples are placed into and removed from ovens, water baths, incubators and other equipment shall be documented if a time limit is required by the method
62	Are samples incubated at 44.5 ± 0.2ºC? [SM 9222 D-2006 (2) (d)]			Incubate for 24 ± 2 h at 44.5 ± 0.2ºC.
63	Is the time samples are removed from the incubator documented? [15A NCAC 2H .0805 (a) (7) (F)]			The date and time that samples are placed into and removed from ovens, water baths, incubators and other equipment shall be documented if a time limit is required by the method.
64	Are appropriate colonies counted? [SM 9222 D-2006 (2) (e)]			Colonies produced by fecal coliform on bacteria on M-FC medium are various shades of blue. Non-fecal Coliform colonies are gray to cream-colored. SM Online: Typical thermotolerant (fecal) coliform colonies are a couple of millimeters in diameter, rather flat, dark cobalt blue colonies. If your water bath temperature is on the low side of the range (e.g. 44.2-44.3 deg C), you may get some mucoid, light blue, or cream-colored colonies which usually do NOT confirm in EC broth. The dark blue colonies typically are confirmed as thermotolerant (fecal) coliforms.
65	What type of microscope is used for counting colonies? [SM 9222 D-2006 (2) (e) and SM 9030 B-2006 (5) (b)] **ANSWER:**			Count colonies with a low-power (10 to 15x magnification) binocular wide-field dissecting microscope or other optical device.
66	How is the density of CFU calculated? [SM 9222 D-2006 (3) (a) and SM 9222 B-2006 (5)] **ANSWER:**			CFU/100ml = $\frac{\text{coliform colonies counted x 100}}{\text{mL sample filtered}}$

67	Are results reported according to the NC WW/GW LC Fecal Coliform Reporting policy document?			See Division document on how to calculate data on the DMR at: http://deq.nc.gov/about/divisions/water-resources/water-resources-permits/wastewater-branch/npdes-wastewater/forms-documents
	COLONY VERIFICATION	**LAB**	**SOP**	**EXPLANATION**
68	Are at least 10 blue colonies verified per month? [40 CFR 136.3 Table IA; footnote 30]			On a monthly basis, at least ten blue colonies from the medium must be verified using Lauryl Tryptose Broth and EC Broth, followed by count adjustment based on these results; and representative non-blue colonies should be verified using Lauryl Tryptose Broth. Where possible, verification should be done from randomized sample sources. If samples do not routinely produce 10 or more blue colonies, verify the first sample during the month which produces blue colonies, regardless of the number. Adjust sample result accordingly. If no samples during the month produce plates with blue colonies, verify 10 colonies from the culture positive. Count adjustments from the culture positive are not to be applied to sample results. **See the Colony Verification Technical Assistance document attached to the end of this checklist.**
69	Are ten LTB fermentation tubes inoculated with ten blue fecal coliform colonies from a single sample? [SM 9020 B-2005 (10) (b) (2)]			Colonies must be from a single sample but may be collected from multiple filters from that sample.
70	Is the inoculating instrument sterilized between each colony/inoculation? [SM 9222 B-2006 (4) (f) (1)]			
71	Are LTB fermentation tubes incubated at 35 ± 0.5°C for 24 ± 2 hours? [SM 9221 B-2006 (2) (b) (2)]			
72	If a water bath is used, is sufficient water depth maintained to immerse LTB tubes to the upper level of the medium? [SM 9221 E-2006 (1) (b) (2)]			
73	After incubation, is each LTB tube swirled gently and examined for growth and/or gas production and the results documented? [SM 9221 B-2006 (2) (b) (2)]			After 24 ± 2 h swirl each tube or bottle gently and examine it for growth, gas, and/or acidic reaction (shades of yellow color) and, if not gas or acidic reaction is evident, reincubate and reexamine at the end of 48 ± 3 h. Record presence or absence of growth, gas, and/or acid production. If the inner vial is omitted, growth with acidity (yellow color) signifies a positive presumptive reaction.
74	If no gas is evident, are LTB tubes re-incubated at 35 ± 0.5°C incubated and additional 24 hours and re-examined for growth or gas production after a total of 48 ± 3 hours? [SM 9221 B-2006 (2) (b) (2)]			
75	For any LTB tubes that exhibit growth and/or+ gas production, are those inoculated into EC medium? [SM 9221 E-2006 (1) (b) (1)]			Gently shake or rotate fermentation tubes or bottles showing gas, growth, or acidity. Using a sterile 3- or 3.5-mm-diam loop or sterile wooden applicator stick, transfer growth from each presumptive or confirmed fermentation tube or bottle to EC broth (see Section 9221B.3).
76	Are EC fermentation tubes incubated within 30 minutes of inoculation? [SM 9221 E-2006 (1) (b) (2)]			Place all EC tubes in a water bath within 30 min after inoculation.
77	Are EC tubes incubated in a water bath at 44.5 ± 0.2°C for 24 ± 2 hours? [SM 9221 E-2006 (1) (b) (2)]			Incubate inoculated EC broth tubes in a water bath at 44.5 ± 0.2°C for 24 ± 2 h.
78	Is sufficient water depth maintained in the waterbath incubator to immerse EC tubes to the upper level of the medium? [SM 9221 E-2006 (1) (b) (2)]			Maintain sufficient water depth in the water bath incubator to immerse tubes to the upper level of the medium.
79	After incubation, is each EC tube examined for growth and gas production and the results documented? [SM 9221 E-2006 (1) (c)]			
80	Are LTB and EC tubes inoculated sequentially at the same time from a single colony to reduce the time of verification analysis? [SM 9222 D-2006 (2) (f)]			

81	When LTB and EC tubes are inoculated at the same time, and only the LTB tubes produce gas, are fresh tubes of EC medium inoculated from the LTB growth and incubated for an additional 24 ± 2 hours?			Based upon EPA Region 4 guidance.
82	If any blue colonies tested do not produce gas, is the colony count of the plate adjusted by the percentage of negative EC medium fermentation tubes prior to reporting results? [40 CFR 136.3 Table IA; footnote 30]			On a monthly basis, at least ten blue colonies from the medium must be verified using Lauryl Tryptose Broth and EC Broth, followed by count adjustment based on these results; and representative non-blue colonies should be verified using Lauryl Tryptose Broth. Where possible, verification should be done from randomized sample sources. For example, if one of ten EC tubes are negative, multiply colony count of the plate by 90% (0.90) and report the result rounded to whole numbers. If a laboratory finds a low percentage of verification with a certain water supply or matrix, another test method must be chosen. [SM 9020 B-2005 (10)]
	QUALITY ASSURANCE	**LAB**	**SOP**	**EXPLANATION**
83	Is a consumables test performed whenever new media, pads or membrane filters are put into use? [NC WW/GW LC Policy]			Before a new lot of consumable materials are used for the Fecal Coliform MF method, those materials must be tested and compared to those currently in use to ensure they are reliable. Consumable materials included in this requirement are: membrane filters and/or pads (often packaged together) and media. It is recommended that only one consumable be tested at a time. At a minimum, make single analyses on five positive samples that will yield 20-60 colonies for both the current lot and the new lot. There are two options for determining acceptance of results: Option 1: Follow the acceptance criteria described in Standard Methods 9020 B-2005 5. (*f)* (2) (a) and (b). Option 2: Compare the average colony count of each five-sample set and evaluate against your routine sample duplicate acceptance criterion. This is not required for reagent water.
84	Is comparability data on file to show that results obtained by the membrane filter method for chlorinated effluents are comparable to those obtained with the multiple tube method? [SM 9222 D-2006] **Not required at this time.**			Since the MF test has been the primary regulatory method used for years in NC this will not be required at this time. Also, with the current discharge limits for TRC in place at most NPDES facilities, TRC is generally not found at levels that would significantly impact the MF test. This decision is also based upon the language found in 9020 regarding costs and time required, 40 CFR 136.3 Table IA footnote #5 states: Because the MF technique usually yields low and variable recovery from chlorinated wastewaters, the Most Probable Number method will be required to resolve any controversies.
85	Are lot numbers of applicable consumable materials documented? [15A NCAC 2H .0805 (a) (7) (K) and NC WW/GW LC Policy]			This includes media, filters, pads and dishes. NC WW/GW LC Policy states: All chemicals, reagents, standards and consumables used by the laboratory must have the following information documented: Date received, Date Opened (in use), Vendor, Lot Number, and Expiration Date (where specified). Consumable materials such as pH buffers, lots of pre-made standards and/or media, solids and bacteria filters, etc. are included in this requirement.

86	Are Ultra-Violet (UV) lamps used for sterilization of filtration equipment? [SM 9020 B-2005 (4) (l)] If not, skip to question 90.			
87	Are UV lamp bulbs cleaned monthly with a soft cloth moistened with ethanol? [SM 9020 B-2005 (4) (l)]			Ultraviolet lamps: Disconnect lamps monthly and clean bulbs with a soft cloth moistened with ethanol.
88	Are UV lamp bulbs tested quarterly with an appropriate UV light meter? [SM 9020 B-2005 (4) (l)]			**Test lamps quarterly with an appropriate UV light meter.**
89	Are UV lamp bulbs replaced if the output is less than 70% of the original? [SM 9020 B-2005 (4) (l)]			Replace bulbs if the output is less than 70% of the original.
90	Is agar refrigerated in the dark and discarded after 2 weeks? [SM 9222 D-2006 (1) (a) and SM 9020 B-2005 (5) (j) (4)]			Purchased prepared media may be used until manufacturer's expiration date. See #19
91	Is broth refrigerated in the dark and discarded after 96 hours? [SM 9222 D-2006 (1) (a) and SM 9020 B-2005 (5) (j) (4)]			Purchased prepared media may be used until manufacturer's expiration date. See question #19
92	Is the autoclave capable of reaching 121ºC? [SM 9030 B-2006 (3)]			Use of pressure cooker is not recommended 9030 B-2006 4 (h). While a specified pressure is not required to be met, it is required to document the pressure. Determining factor in sterilization is the 121 ºC not the pressure. Must have pressure to reach this required temperature. SM 9030 B-2006 (3) states autoclave must be able to reach 121 °C within 15 minutes. This is only required if autoclave is used to sterilize media – cannot sterilize MF media in autoclave – only MPN media, so this is applicable to the monthly fecal colony verification.
93	Is an autoclave log maintained? [SM 9020 B-2005 (4) (h)] and [15A NCAC 2H .0805 (a) (7) (l)]			SM states: Record items sterilized and sterilization temperature along with total run-time (exposure to heat), actual time period at sterilization temperature, set and actual pressure readings, and initials of responsible person for each run cycle. This means three times must be recorded (start time, time it reaches set point and end time. Alternatively, verify the cycle time at operating temperature and pressure annually and document cycle start time and length each day of use. Also, document that autoclave tape is positive for adequate temperature. May just have column for autoclave tape positive and check the box.
94	Is heat indicating tape used with all materials each sterilizing cycle and its use documented? [SM 9020 B-2005 (4) (h)]			SM states: The additional use of a steam indicator for each cycle is a practical and quick method to show minimum exposure conditions were met. **Use heat-indicating tape to identify supplies and materials that have been sterilized.** Use of autoclave tape is required. Each item per autoclave sterilization batch does not have to have tape on it. A representative item may have tape placed on it to demonstrate adequate sterilization.
95	Is the autoclave temperature checked weekly with a maximum registering thermometer and documented? [SM 9020 B-2005 Table 9020:I] and [15A NCAC 2H .0805 (a) (7) (E)]			Must distinguish between daily autoclave temperature and reading from the weekly maximum registering thermometer (MRT) placed inside autoclave in documentation. Annual calibration of the maximum registering thermometer is not required.
96	Is glassware not in metal containers sterilized in a 170ºC oven for a minimum of 1 hour? [SM 9040-2006]			Sterilize glassware, except when it is in metal containers, by dry heat for 2 to 4 hours at a temperature of 170ºC.

97	Is glassware in metal containers sterilized in a 170ºC oven for a minimum of 2 hours? [SM 9040-2006]			Sterilize glassware in metal containers at 170ºC for not less than 2 hours.
98	How are sample bottles sterilized? [SM 9020 B-2005 Table 9020: IV] and [SM 9040-2006] **ANSWER:**			Sterilize bottles not made of plastic as above. Glass or plastic sample bottles may be sterilized in an autoclave at 121ºC for 15 min. Moisture present in bottles after autoclaving may be removed by placing in a drying oven at 100ºC for 10 to 15 min. For plastic bottles, loosen caps before autoclaving to prevent distortion. Many labs use sterilized bottles or sample bags.
99	Are laboratory sterilized bottles checked for sterility? [SM 9020 B-2005 (5) (d)]			SM States: Minimally test for sterility one sample bottle per batch sterilized in the laboratory or one sample bottle per lot of purchased as pre-sterilized, or at a set percentage such as 1 to 4%. Autoclave tape alone not adequate – need to add sterile dilution/rinse water to bottle and analyze. We will except Certificate of Analysis for store bought bottles or sample bags in lieu of the above testing.
100	Is the incubator temperature documented twice daily (morning and afternoon)? [SM 9020 B-2005 (4) (n)]			When incubator is in use, monitor and record calibration-corrected temperature twice daily. Although the method requires the use of a full-immersion thermometer, this has been acknowledged by the Standard Methods Committee as an error and partial immersion thermometers are also acceptable to use.
101	Is the thermometer/temperature monitoring device graduated in 0.1°C increments? [SM 9030 B-2006 (12)]			Be sure to check thermometer in water bath to ensure tip is not sitting on bottom of incubator. Check thermometer immersion type (total vs. partial) and line.
102	Is the thermometer/temperature monitoring device calibrated quarterly? [SM 9020 B-2005 4 (a) and 15A NCAC 2H .0805 (a) (7) (N) (iii)]			Rule: Digital temperature-measuring devices and temperature-measuring devices used in incubators shall be verified at the temperature of use every three months against a Reference Temperature-Measuring Device and their accuracy shall be corrected. SM states: Annually or, preferably, semiannually check accuracy of all working temperature-sensing devices, such as liquid-in-glass thermometers, thermocouples, and temperature-recording instruments at the use temperature against a certified National Institute of standards and Technology (NIST) thermometer or one traceable to NIST and conforming to NIST specifications. Record calibration results, along with the date and technician's signature, in a quality control logbook. Mark the necessary calibration correction factor on each temperature measuring device so that only calibrated- corrected temperature values are recorded. Verify accuracy of the reference certified thermometer as specified on the certificate of calibration or at least every 5 years. Make sure the thermometer used in the water bath or the thermometer used to calibrate it, is accurate to 0.1°C.
103	Is the temperature correction posted? [SM 9020 B-2005 4 (a)]			See above
104	Is a culture positive analyzed with each batch of prepared media? Each week for purchased ready-to-use media? [SM 9020 B-2005 (9) (b)] [NC WW/GW LC Policy]			SM Table 9020:I. states: media – Check performance with + and - culture controls – Each batch or lot SM 9020 B-2005 (5) (j) (4) states: For prepared ready-to-use media with a manufacturer's expiration date greater than noted in the table, have manufacturer supply evidence of media quality for that extended period of time. Verify usability weekly by testing recoveries with known densities of culture controls that will also meet QC check requirements. SM 9020 B-2005 (5) (j) (7) states: Quality control of purchased prepared media – Test each new lot for

				sterility and with positive and negative control cultures. For purchased prepared media which have a longer shelf-life than those prepared in the laboratory, perform these tests more frequently. **Due to the reasons given in question #1, NC WW/GW LC will require a culture positive (no culture negative) <u>once per week for purchased premade media</u> and <u>once per prepared batch for laboratory prepared media</u> in lieu of the above requirements at this time.** NC WW/GW LC Policy: A culture positive must be analyzed with each batch of prepared media and once per week for purchased ready-to-use media
105	Are the culture positive plates countable? [NC WW/GW LC Policy]			A culture positive must be analyzed with each batch of prepared media and once per week for purchased ready-to-use media. A sample volume that yields a countable plate must be analyzed so that individual colonies may be verified to have proper morphology (i.e. color, shape, size, surface appearance). **Often culture positives are TNTC. The analyst must set a volume that yields a countable plate. This does not necessarily mean in the range of 20-60 CFU. The point of a culture positive is beyond just the ability to grow colonies but also to be able to discern individual colonies for proper morphology – that is color, shape, surface appearance, size etc. A sample (e.g., stream samples) may also serve as a culture positive if identified as such.**
106	Are sterility checks (blanks) performed on the entire process at the beginning and end of each filtration series of samples, using 20 to 30 ml of sterile reagent or dilution water as the sample? [SM 9222 D-2006 (1) (a)]			SM states: For membrane filter tests, check the sterility of the entire process by using sterile reagent or dilution water as the sample at the beginning and end of each filtration series of samples and test for growth. This means: Check for Coliform contaminations at the beginning and end of each filtration series by filtering 20 to 30 ml of dilution or rinse water through filter. These are blanks. If more samples are analyzed after 30 minutes of completing an analytical batch the funnels must be sterilized and beginning and end blanks analyzed with the new sample batch.
107	If there is an interruption of more than 30 minutes in the filtration sequence, are new sterilized funnels used and the sterility test repeated? [SM 9020 B-2005 (9) (d)]			With processing interruption of more than 30 min use new sterilized funnels and repeat sterility test.
108	What corrective action is taken when contamination is apparent? [15A NCAC 2H .0805 (a) (7) (B)] **ANSWER:**			Samples results must be qualified.
109	Are single analyst counts on one or more positive samples repeated at least monthly and the results within 5% difference? [SM 9020 B-2005 (9) (a)]			SM states: For routine performance evaluation, repeat counts on one or more positive samples at least monthly, record results, and compare the counts with those of other analysts testing the same samples. **Replicate counts for the <u>same analyst</u> should agree within 5% (within analyst repeatability of counting)** and those between analysts should agree within 10% (between analysts reproducibility of counting). If they do not agree, initiate investigation and any necessary corrective action. See 9020 B-2006 (13) (b) for a statistical calculation of data precision. **Labs that typically have very few or no colonies on their effluent sample may use their culture positive for this.**

110	When there are multiple analysts in the laboratory, are counts on one or more positive samples repeated at least monthly and the results agree within 10% difference of the other analysts? [SM 9020 B-2005 (9) (a)]			SM states: For routine performance evaluation, repeat counts on one or more positive samples at least monthly, record results, and compare the counts with those of other analysts testing the same samples. **Replicate counts <u>between analysts</u> should agree within 10% (between analysts' repeatability of counting)**. If they do not agree, initiate investigation and any necessary corrective action. See 9020 B-2005 (13) (b) for a statistical calculation of data precision. Note: Counts between analysts do not have to be performed on the same day. In this case, the plate must be refrigerated. The average of each single analyst count may be used for the multi-analyst comparison. **Labs that typically have very few or no colonies on their effluent sample may use their culture positive for this.**
111	Are at least five percent of all samples analyzed in duplicate to document precision? Or, if analyzing less than 20 samples per month, is at least one duplicate analyzed per month? [15A NCAC 2H .0805 (a) (7) (C)]			**At this time, we will follow our Rules for duplicate frequency.** Except where otherwise specified in an analytical method, laboratories shall analyze five percent of all samples in duplicate to document precision. Laboratories analyzing fewer than 20 samples per month shall analyze one duplicate during each month that samples are analyzed.
112	What is the acceptance criterion for duplicates?[15A NCAC 2H .0805 (a) (7)] and [15A NCAC 2H .0805 (a) (7) (A)] **ANSWER:**			Unless specified by the method or this Rule, each laboratory shall establish performance acceptance criteria for all quality control analyses. If the laboratory has different acceptance criteria for plate counts with greater than and less than 20 CFUs, they must establish which acceptance criterion will be used to evaluate the duplicates (e.g., plates with 18 and 22 CFUs). Supporting records shall be maintained as evidence that these practices are being effectively carried out. The quality control document shall be available for inspection by the State Laboratory. If an RPD limit between colony counts is used, the mean in the calculation should be an arithmetic mean. If reporting an average of duplicate results(instead of reporting both individual results), the DWR Water Quality Permitting Section has stipulated that it must be the geometric mean; not the arithmetic mean. Keep in mind we are not talking about reporting the duplication of one dilution out of a series of dilutions. Those would be figured into the single result for that sample and not independently reported. This only applies if the entire sample was duplicated or more than one sample was collected in single day.
113	What corrective action does the laboratory take if the duplicate sample results are outside of established control limits or method precision limits? [15A NCAC 2H .0805 (a) (7) (B)] **ANSWER:**			
114	Is reagent water testing being performed? [NC WW/GW LC Policy]			At a minimum, reagent water used to make dilutions, prepare buffered dilution/rinse water or prepare media must be analyzed at least every twelve months for the following parameters:

				Specific Conductance, Total Organic Carbon, Cadmium, Chromium, Copper, Nickel, Lead, and Zinc. Maximum Acceptable Limits are: Total Organic Carbon < 1.0 mg/L Specific Conductance < 2 µmhos/cm Heavy Metals, single element < 0.05 mg/L Heavy Metals, Total of specified elements < 0.10 mg/L If the facility is using vendor purchased reagent water or dilution/rinse water, this testing is not required as long as the Certificate of Analysis from the manufacturer meets these requirements and is kept on file.
115	Is the data qualified on the Discharge Monitoring Report (DMR) or client report if Quality Control (QC) requirements are not met? [15A NCAC 2H .0805 (a) (7) (B)]			If the sample cannot be reanalyzed, or if the quality control results continue to fall outside established limits or show an analytical problem, the results shall be qualified as such. If data qualifiers are used to qualify samples not meeting QC requirements, the data may not be useable for the intended purposes. It is the responsibility of the laboratory to provide the client or end-user of the data with sufficient information to determine the usability of the qualified data.

Fecal Coliform-MF is a method-defined parameter.

Additional Comments:

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Inspector: _____Date:_____

The following Fecal Coliform parameter codes have been removed from the allowable parameter list for DMRs: 31613 and 31615.

The only allowable Fecal Coliform parameter code in BIMS is 31616. This is regardless of technology (e.g., Colilert-18, MF, MPN). The parameter descriptor has been modified to "Coliform, Fecal".

The allowable units of measure for 31616 are: 1. Most Probable Number (MPN) per 100mL (MPN/100mL); 2. Number per 100mL (#/100mL); and 3. Colony Forming Units per 100mL (CFU/100mL).

Testing of Consumable Materials for Fecal Coliform MF Method (NC WW/GW LC Policy 02/07/2017)

Before a new lot of consumable materials are used for the Fecal Coliform MF method, those materials must be tested and compared to those currently in use to ensure they are reliable. Consumable materials included in this requirement are: membrane filters and/or pads (often packaged together) and media. **It is recommended that only one consumable be tested at a time.**

At a minimum, make single analyses on five positive samples that will yield 20-60 colonies for both the current lot and the new lot.

There are two options for determining acceptance of results:

Option 1:

Follow the acceptance criteria described in Standard Methods 9020 B 5. *f* 2) a) and b).

Option 2:

Compare the average colony count of each five-sample set and evaluate against your routine sample duplicate acceptance criterion.

<u>The following is provided as guidance in performing the required testing followed by evaluation according to the duplicate acceptance criterion option.</u>

Let's say you got a new batch of membrane filters in. We will call the currently used filters lot #1 and the new filters lot #2.

1. Select a culture positive sample.

What you want is something that will yield 20-60 colonies. This may be a stream sample or a sample taken somewhere within the waste treatment plant. If the concentration is high enough that greater than 60 colonies are obtained when 1 mL is filtered, then the solution is too strong and must be diluted. Any time a sample is diluted be sure it is done with the BUFFERED dilution water used for rinsing the funnels.

2. Test the culture positive to determine the appropriate volume to use.

When collecting the culture positive sample do not think about it as a sample. You do not have to be concerned with a sterile sample bottle or 6 hour hold time. Collect enough sample so that you have plenty to work with, probably more than your normal fecal bottle holds. Set a series of dilutions using the currently used materials (filter lot #1). Do not use the materials you want to prove are OK at this point. All you are trying to do is to determine the volume of sample that will yield 20-60 colonies. Put the rest of the sample in the refrigerator. For example:

Volume used	Colonies obtained on lot #1 filters
50 ml	TNTC
25 ml	138
10 ml	50
5 ml	22
1 ml	4

Based on this preliminary testing it appears that a 10 ml volume would probably be appropriate to use and will yield the desired 20-60 colonies. Remember, when you do the actual consumable test, the culture positive sample will be 24 hours old and the results you obtain may be lower than the initial results yielded, but not so significantly lower as to change your dilution choice. It is better to have your initial results on the high side of the 20-60 range for this reason. In this example the 5 ml volume would probably be too low and would likely yield less than 20 colonies the next day.

3. Perform the consumable test

Once you determine the appropriate volume, in this case 10 mls, take the remaining culture positive sample from the refrigerator, bring to room temperature and set five 10 ml plates with the currently used filters (lot #1) and five 10 ml plates with the new filters (lot #2).

4. Determine acceptability of new material

For example:

Lot #1-current filters	Colonies obtained on lot #1 filters
10 ml	48
10ml	45
10 ml	50
10 ml	44
10 ml	43
Average:	46

Lot #2-new filters	Colonies obtained on lot #2 filters
10 ml	40
10ml	45
10 ml	38
10 ml	46
10 ml	37
Average:	41

When determining the acceptability of the new material, compare the average of the five replicates for lot #1 to the average of the five replicates for lot #2; that is 46 vs. 41 colonies. The comparison of results must adhere to your current acceptance criteria used for your Fecal Coliform **duplicates**. If the test and reference materials check within what you have determined is acceptable for duplicates of samples, the test material would be considered acceptable to use. This may be a calculated acceptance criterion based on 3 times the standard deviation of the mean or a set value like 20% RPD. No matter how you determine your duplicate acceptance criterion make sure you **use colony counts not final calculated values** in doing this. Other factors to consider when determining if a new material is suitable include:

Are the colonies obtained typical, that is normal looking blue colonies?
Are the colonies evenly distributed across the membrane surface?
Are there an unusual number of non-typical colonies present?
Is there a pattern to the colony recoveries? For example, are all the plates for the test materials significantly lower in counts than the reference lot?

It is recommended that new consumables be tested as soon as possible after receipt to avoid problems if the materials are not acceptable. Once you determine that the new material is acceptable to use; you may begin to do so. Document the date the new lot # is put into use.

May 23, 2019

FECAL COLIFORM REPORTING

The following criteria are to be used in obtaining and reporting fecal coliform data:

Standard Methods suggests analyzing samples by filtering three different volumes (diluted or undiluted) depending on the bacterial density. Each laboratory must filter multiple dilutions of the sample in order to obtain plates containing 20 to 60 fecal coliform colonies. It is **recommended** that sampling containers of at least 250 ml be used in order to collect sufficient volume to meet method criteria. The requirements for calculating values are given in Standard Methods 9222 D-2006, and EPA Microbiological Methods for Monitoring the Environment, Water, and Wastes, EPA/600/8-78/017. 1978. US EPA. Page 124. The following is a compilation of these requirements to be used in calculating the fecal coliform count per 100 ml of sample for compliance with NC WW/GW Laboratory Certification.

ALL RESULTS MUST BE REPORTED IN WHOLE NUMBERS.

(1) **Countable Membranes with 20-60 Blue Colonies:** Calculate the fecal coliform results from membrane filters within the ideal counting range of 20-60 blue colonies using the general formula:

$$\frac{\text{Number of colonies counted} \times 100}{\text{volume of sample filtered in ml}} = \text{Fecal coliform colonies per 100 ml}$$

If more than one filter (including a 100 ml sample volume) has a count in the acceptable range, calculate the values in counts/100 ml for each filter in the countable range and average.

(2) **Countable Membranes with less than 20 Blue Colonies:** If all counts are below the lower limit (20) of the ideal counting range:
(a) Select the count most nearly acceptable and compute the count using the general formula. Report the count as an Estimated Count per 100 ml: **or**

(b) Total the counts on all filters and report as number per 100 ml. For example, if 50, 25, and 10 ml portions were examined, and counts were 15, 6, and 0 coliform colonies respectively, calculate results as follows and report the count as 25 colonies per 100 ml.

$$\frac{(15 + 6 + 0) \text{ counts} \times 100}{50 + 25 + 10 \text{ ml}} = 25 \text{ colonies per 100 ml}$$

(3) **Membranes with No Colonies:** If counts from all filters are zero, report the count for the fecal coliform as a less than (<) value. Calculate the number of colonies per 100 ml that would have been reported if there had been one colony on the filter representing the largest filtration volume. For example, sample volumes of 25, 10 and 2 ml produced colony counts of 0, 0 and 0, respectively. The count would be reported as <4 colonies per 100 ml. If a 100 ml sample was analyzed report as < 1/100 ml.

$$\frac{<1 \text{ counts} \times 100}{25 \text{ ml}} = <4 \text{ colonies per 100 ml}$$

(4) **Countable Membranes with more than 60 Colonies:** If all filter counts are above the upper limit (60), but countable, calculate the count from the smallest volume filtered and report as a greater than (>) value. For example, if 10, 5, and 1 ml portions of samples were examined, and counts were TNTC, 310, and 95 coliform colonies respectively, calculate results as follows and report the count as >9500 colonies/100 ml.

$$\frac{>95 \text{ counts} \times 100}{1 \text{ ml}} = >9500 \text{ colonies per 100 ml}$$

(5) **Uncountable Membranes**: For uncountable filters with more than 60 colonies or "Too numerous to Count" (TNTC), use 60 colonies as the basis of calculation with the smallest filtration volume and report as a greater than value. For example, sample volumes of 10, 1.0 and 0.1 ml all produced too many colonies to show separated colonies and the laboratory bench sheet showed TNTC. The count would be reported as >60,000 colonies per 100 ml.

$$\frac{>60 \text{ counts} \times 100}{0.1 \text{ ml}} = >60{,}000 \text{ colonies per 100 ml}$$

(6) **If the Filters for a sample have counts of both >60 and <20, but none in the 20-60 range:** Use all countable filters and calculate as in (2) (b) above.

(7) **Anomalies:** The above requirements are to be used except when an abnormality occurs in the analysis of a sample. When abnormalities occur, analysts must use their best judgment in selecting the proper value to report.

(8) **Reporting Data for Multiple Samples in a Single Day or Duplicate Samples:** When only one dilution is duplicated, that would be included in the sample calculation and not reported as separate result. When the entire dilution scheme for a sample is duplicated or if more than one sample is collected in a single day, all results must be reported. The DWR Water Quality Permitting Section has directed that those results may be reported discretely, or a as a geometric mean. If reported discretely, the highest value would go in the daily cell and the rest would go in the comment section. If the geometric mean is reported, the comment section must indicate the reported value is a geometric mean of either a duplicate sample or of multiple samples collected on that day.

Fecal Coliform (Membrane Filter) Colony Verification Technical Assistance

With regards to Fecal Coliform by Standard Methods, 9222 D-2006, the Code of Federal Regulations, Title 40, Part 136; Federal Register Vol. 82, No. 165, August 28, 2017; 136.3. Table IA, Footnote 30 and Table IH, Footnote 27 state: *On a monthly basis, at least ten blue colonies from the medium must be verified using Lauryl Tryptose Broth and EC broth, followed by count adjustment based on these results; and representative non-blue colonies should be verified using Lauryl Tryptose Broth. Where possible, verifications should be done from randomized sample sources.*

Standard Methods, 9020 B-2005. (10) states: *Verification is a general process used to determine whether the microbiological analytical method is performing as expected to provide reliable data. If a laboratory finds a low percentage of verification with a certain water supply or matrix, another test method must be chosen. To determine false negatives, pick representative atypical colonies of different morphological types and verify as in Presumptive Phase [Lauryl Tryptose Broth], Section 9221B.*

If samples do not routinely produce 10 or more blue colonies, verify the first sample during the month which produces blue colonies, regardless of the number. Adjust sample result accordingly.

If no samples during the month produce plates with blue colonies, verify 10 colonies from the culture positive. Count adjustments from the culture positive are not to be applied to sample results.

Colony verification may not be subcontracted to other laboratories.

Reagents:

Lauryl Tryptose Broth (LTB) - Ref: SM 9221 B-2006. (2) (a). Equivalent to Lauryl Sulfate Broth. Do not use LTB with MUG.

EC Medium - Ref: SM 9221 E-2006. (1) (a). Do not use EC Medium with MUG.

LTB and EC Medium may be prepared from commercially available dehydrated medium or purchased already prepared in fermentation tubes containing inverted Durham tubes.

Equipment and Supplies:

Fermentation tubes and Durham tubes. Ref: SM 9030 B-2006. (17).

Inoculating equipment: Use wire loops made of 22- or 24-gauge nickel alloy or platinum-iridium for flame sterilization. Use loops at least 3 mm in diameter. Single-service hardwood or plastic applicators, 0.2 to 0.3 cm in diameter and at least 2.5 cm longer than the fermentation tube, also may be used. Sterilize wooden applicators by dry heat and plastic applicators by autoclave, while stored in glass or other non-toxic containers. Prepackaged disposable plastic loops also are available for ready use. Ref: SM 9030 B-2006. (18)

Incubator at 35 ± 0.5 °C: an air incubator with 60% relative humidity or a water bath incubator may be used. Ref: SM 9030 B-2006. (1).

Incubator at 44.5 ± 0.2 °C: a water bath is required. Ref: SM 9030 B-2006. (1).

Preparation of Media:

1. Prepare LTB and EC Medium according to manufacturer's instructions, or by following *Standard Methods* if commercially-prepared medium is not used.
2. Before sterilization, dispense – in sterilized fermentation tubes with an inverted vial (Durham tube) – sufficient medium to cover the inverted vial at least one-half to two-thirds after sterilization.
3. Loosely cover tubes with metal or heat-resistant plastic caps.
4. Autoclave at 121 °C for 12-15 minutes with the exhaust set to slow.
5. After autoclaving, ensure that the inverted vials are free of air bubbles and discard any that contain air bubbles.
6. Check and document pH of media after sterilization. LTB should be 6.8 ± 0.2 S.U. and EC Medium should be 6.9 ± 0.2 S.U. If not, adjust pH using 1*N* NaOH or 1*N* HCl that has been filtered and sterilized. If the pH is more

than 0.5 S.U. outside of the specified pH, discard and determine why (e.g., incorrect preparation or abnormal pH of reagent water).

7. Allow tubes to cool to room temperature and tighten caps on the tubes.
8. Follow manufacturer's recommendations for storage and expiration of prepared media. If the media is refrigerated, allow to warm to room temperature (20°C) before use. Discard any tubes that contain air bubbles.

Colony Verification Procedure:

Verification testing takes 48-72 hours. Take this into consideration when preparing for analysis. An alternative procedure that may reduce the testing time to 24 hours is described in #9 below.

1. After performing the colony count for membrane filtration, using a random sample source if possible, inoculate the LTB using a sterilized inoculating instrument (e.g., a sterile 3- to 3.5-mm -diameter loop or sterile wooden applicator stick). This is performed by scraping each colony with the inoculating instrument, dipping it into the media and gently swirling it. The inoculating instrument must be sterile for each individual colony.
2. Inoculate ten LTB fermentation tubes with ten typical blue fecal coliform colonies from a single sample, sterilizing the inoculating equipment between each inoculation. It is also recommended that representative non-blue colonies also be inoculated in LTB fermentation tubes to determine false negatives.
3. Incubate the LTB fermentation tubes at 35 ± 0.5 °C for 24 ± 2 hours. If using a water bath, maintain a sufficient water depth to immerse tubes to the upper level of the medium.
4. Swirl each tube and examine it for gas production. Document results.
5. If no gas is evident, continue incubation and re-examine at the end of 48 ± 3 hours.
6. For any tubes that exhibit gas production, inoculate the EC Medium by dipping the inoculating instrument into the LTB and transferring a small portion to the EC Medium.
7. Incubate the EC fermentation tubes within 30 minutes of inoculation at 44.5 ± 0.2 °C for 24 ± 2 hours. To maintain this temperature range, a water bath is required for incubation of EC Medium. Maintain a sufficient water depth to immerse tubes to the upper level of the medium.
8. Swirl each tube and examine for gas production. Document results.
9. It is permissible to inoculate tubes of LTB and EC Medium sequentially at the same time from a single colony to reduce the time of the analysis. This is performed by scraping the colony with the inoculating equipment, inserting it into the LTB and gently swirling it, and then inserting it into the EC Medium and gently swirling it. If both media are inoculated at the same time, but only the LTB tube produces gas after the incubation period, fresh tubes of EC Medium must be inoculated from the LTB and incubated for an additional 24 ± 2 hours.

Interpretation:

Gas production in both the LTB and EC Mediums confirms that the colony is a fecal (thermotolerant) coliform. The media will also become turbid or cloudy when gas is produced. If any blue colonies are not confirmed to be fecal (thermotolerant) coliforms, adjust the colony count of the plate by the percentage of negative EC Medium fermentation tubes prior to reporting the results. For example, if one of ten EC tubes are negative, multiply colony count of the plate by 90% (0.90) and report the result rounded to whole numbers.

If any non-blue colonies that are tested produce gas in the LTB, they may be fecal (thermotolerant) coliforms. It is recommended that this be confirmed by inoculating EC Medium and incubating for 24 hours.

Laboratories must choose another approved method for analysis if a low percentage of verification is found for a specific sample site.

It is recommended that the laboratory take notes about the size, shape and color of colonies that are verified, especially if there are any abnormalities. Pictures of the plates may also help the laboratory maintain this information.